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Effects of Microtubule Modulators on HIV-1 Infection of Transformed and Resting CD4 T Cells▽

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Previous studies have observed fluorescently labeled HIV particles tracking along microtubule networks for nuclear localization. To provide direct evidence for the involvement of microtubules in early steps of HIV infection of human CD4 T cells, we used multiple microtubule modulators such as paclitaxel (originally called taxol; 1 μM), vinblastine (1 and 10 μM), colchicine (10 and 100 μM), and nocodazole (10 and 100 μM) to disturb microtubule networks in transformed and resting CD4 T cells. Although these drugs disrupted microtubule integrity, almost no inhibition of HIV-1 infection was observed. Our results do not appear to support an essential role for microtubules in the initiation of HIV infection of CD4 T cells.

As obligate intracellular parasites, viruses frequently utilize the cytoskeleton for entry and intracellular transport (28, 32). The human immunodeficiency virus (HIV-1) appears to be one of the pathogens that rely on the host cytoskeleton, both actin and microtubules, to initiate infection (14, 28, 32). It has been suggested that the cortical actin is directly involved in viral gp120-mediated CD4-CXCR4 clustering, which may facilitate viral entry (7, 18, 19, 29, 33, 41). After membrane fusion, HIV-1 encounters the dense meshwork of the cortical actin that appears to be important for viral reverse transcription (9, 47, 48). The cortical actin itself may also represent a barrier for viral intracellular migration (47, 48), particularly in noncycling resting CD4 T cells, where the actin cortex is less dynamic (48). HIV-1 may utilize both viral proteins, such as gp120 and Nef (1, 10, 11, 37, 48), or host factors such as cofilin and LIMK1 to remodel cortical actin, thereby allowing HIV-1 to overcome this restriction (37, 48). Following migration across the actin cortex, the virus has to travel through the cytoplasm for nuclear localization. Because of the high viscosity of the cytoplasm, it has been suggested that HIV-1 may utilize the microtubule network for intracellular trafficking (4, 27, 50). In general, intracellular movement of macromolecules or organelles within the cytoplasm requires actin microfilaments for short-distance travel and microtubules for long-distance migration. Indeed, using fluorescently labeled HIV-1 particles, multiple studies have shown that the preintegration complex (PIC) of HIV-1 tracks along microtubules and accumulates in the microtubule-organizing center for nuclear localization (4, 27, 50). Nevertheless, concerns have been raised regarding whether these microscopy experiments distinguish replication-competent viruses from mostly noninfectious particles (38). In addition, these previous imaging studies were often undertaken using adherent, immortalized cell lines that have extensive microtubule networks compared to those of primary CD4 T cells. It is interesting to note that the relatively thin cytoplasm in T cells may require only short-distance travel for viral nuclear localization (Fig. 1A). Thus, the biological significance of HIV tracking along microtubules remains a subject of debate. In this article, using pharmacological modulators of microtubules, we investigated the potential role of microtubules in the initiation of HIV-1 infection of T cells.

To provide direct evidence for the involvement of microtubules in the early steps of HIV infection, we used multiple microtubule modulators, such as paclitaxel (originally called taxol; TX), vinblastine (VB), colchicine (CN), and nocodazole (ND) to stabilize or disrupt microtubules in human T cells. TX is a complex diterpene initially identified in screens of compounds with anticancer activity (42). TX binds and stabilizes microtubule polymers, thereby preventing microtubule dissociation (36). VB is a plant alkaloid that binds to tubulin and prevents microtubule assembly at concentrations at or below 10 μM (21, 24, 34, 44). CN binds to tubulin and blocks microtubule formation by stimulating the intrinsic GTPase activity of tubulin (5, 25). The tubulin dimer must be bound to GTP for normal microtubule assembly to occur. Finally, ND disrupts microtubules by binding to β-tubulin and prevents the formation of one of the two interchain disulfide bridges (26).

To verify that these microtubule modulators were in fact disturbing the microtubule network at the applied dosages, we tested them on a human indicator T cell line, Rev-CEM (45). We also tested these drugs on primary human resting CD4 T cells that were purified from peripheral blood by negative depletion (46). For comparison, adherent HeLa cells cultured on poly-L-lysine-coated coverslips were treated in an identical manner. Cells were rested overnight and then treated with the drugs for 2.5 h at the following doses: 1 μM TX, 1 and 10 μM VB, 10 and 100 μM CN, and 10 and 100 μM ND (6, 12, 17). As a control, cells were treated in the identical manner with the solvent dimethyl sulfoxide. (DMSO). After treatments, cells were fixed, permeabilized (Cytofix/Cytoperm, Perm/Wash buffer; BD Biosciences, San Jose, CA), and then stained for confocal microscopy of the microtubule networks. For microtubule staining, cells were incubated with a biotinylated anti-α-tubulin antibody (clone 10D8; Biolegend, San Diego, CA),

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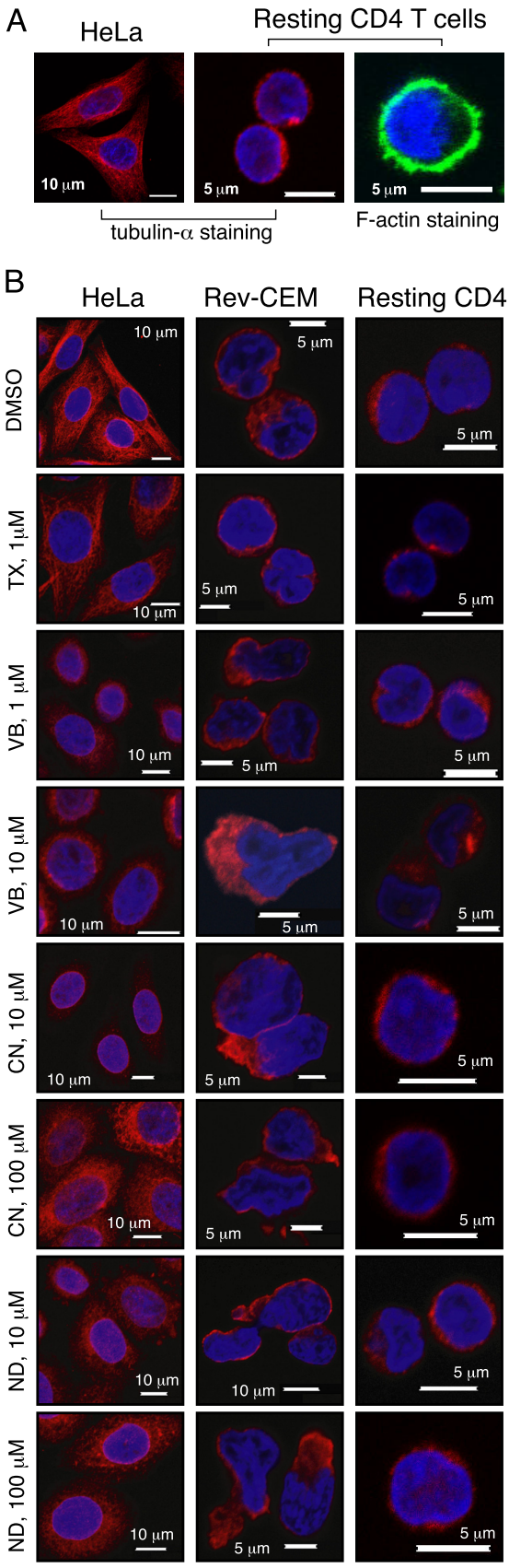

followed by staining with Alexa Fluor 594-labeled streptavidin (Invitrogen, Carlsbad, CA). As shown in Fig. 1B, an extensive microtubule network was observed in HeLa cells. In contrast, Rev-CEM and resting CD4 T cells exhibited uniform round shapes with low levels of microtubule staining. As expected, TX treatment did not result in robust changes in cell shape or the microtubule network of HeLa, Rev-CEM, or resting CD4 T cells. The preservation of microtubule networks was expected in TX-treated cells because TX binds and stabilizes existing microtubule polymers, thereby preventing microtubule dissociation (36). For VB at both dosages (1 and 10 μM), a complete collapse of the microtubule network was observed, with microtubules remaining only near the nucleus in HeLa cells. CN has a mechanism of action similar to that of VB in that it prevents microtubule assembly. In 10 and 100 μM CN-treated HeLa cells, a definite reduction in the number and branching of microtubules was observed. ND disrupts microtubules by binding to β-tubulin and prevents the formation of one of the two interchain disulfide bridges (26). Treatment of HeLa cells with 10 or 100 μM ND resulted in a loss of the elongated microtubule network; it was replaced with shortened microtubules that were condensed and spiral shaped.

For human T cells, treatment of Rev-CEM cells with VB, CN, and ND resulted in cell shape changes characterized by polarized microtubule staining, with cells exhibiting a flattened leading edge and a retracting edge. Treatment of resting CD4 T cells with these drugs also resulted in morphological changes similar to those in Rev-CEM cells but to a lesser degree. This was expected, as resting CD4 T cells have large nuclei, little cytoplasm, and very low levels of microtubule staining.

Next, we tested the requirement for microtubule integrity in the early phases of HIV-1 infection of T cells. Given the high dosages of the drugs used and the expected cytotoxicity, we used the Rev-dependent green fluorescent protein (GFP) indicator T cell line Rev-CEM (45) to measure the inhibition of HIV-1 infection instead of simply using a p24 enzyme-linked immunosorbent assay (ELISA), which by itself is not able to distinguish between general drug cytotoxicity and HIV-specific inhibition (49). Additional advantages of using the cell line Rev-CEM are its high specificity and nonresponsiveness to non-HIV induction. This is distinguishably different from long terminal repeat (LTR)-driving reporter cells that are often leaky and highly responsive to agents stimulating cellular activities. It has been known that mi-

FIG. 1. (A) Comparison of microtubule staining results between HeLa and human CD4 T cells. HeLa cells or resting CD4 T cells from blood were fixed, washed, and then stained with biotinylated anti-α-tubulin antibody, followed by incubation with Alexa Fluor 594-conjugated strepavidin. Cells were mounted in ProLong Gold antifade reagent (Invitorgen) with DAPI (4′,6-diamidino-2-phenylindole) staining of the nucleus (blue) for confocal microscopy. For comparison, resting CD4 T cells were also stained with fluorescein isothiocyanate (FITC)-labeled phalloidin for actin filaments. (B) Confocal microscopy of HeLa, Rev-CEM, and resting CD4 T cells treated with pharmacological microtubule modulators. Cultured cells were treated with 1 μM paclitaxel (TX), 1 μM and 10 μM vinblastine (VB), 10 μM and 100 μM colchicine (CN), or 10 μM and 100 μM nocodazole (ND) (all purchased from Sigma-Aldrich, St. Louis, MO) for 2.5 h, followed by fixation and staining with biotinylated anti-α-tubulin antibody as described for panel A. For a control, cells were also treated in the same manner with DMSO (0.3%, vol/vol) and stained.

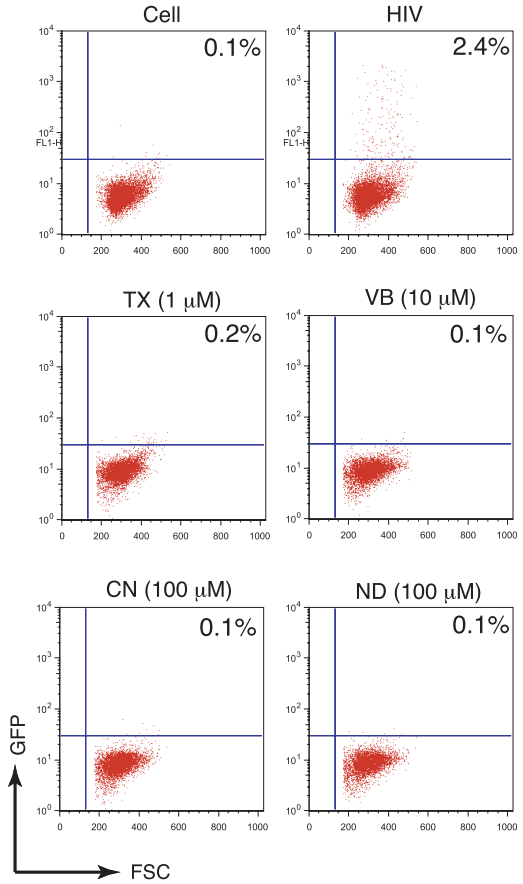

FIG. 2. Microtubule modulators do not stimulate HIV-independent expression from the Rev-dependent GFP indicator cell Rev-CEM. Cells were treated with TX, VB, CN, or ND at the indicated dosages for 2.5 h in the absence of HIV infection. Cells were washed and cultured for 48 h, and then GFP expression was monitored by flow cytometry at 48 h (20,000 cells analyzed per sample). Propidum iodide (PI) was added to the cell suspension prior to flow cytometry. Viable cells were gated based on low PI staining and cell size (FSC, forward scatter). GFP expression within the viable cell population was measured. The GFP percentages are shown. As controls, cells were infected with HIV, which resulted in HIV Rev-dependent GFP expression. Similar drug treatments for 6 h also did not result in GFP expression in the absence of HIV (data not shown).

crotubule inhibitors such as TX and VB are capable of inducing the expression of reporter genes driven by the HIV-LTR promoter (2, 39). However, when tested on Rev-CEM cells, these drugs alone were not capable of stimulating GFP expression (Fig. 2), demonstrating the high stringency of Rev-CEM cells for HIV-dependent reporter expression.

To measure the inhibition of HIV-1 infection by microtubule modulators, Rev-CEM cells were pretreated with the drugs, either for 30 min (Fig. 3B) or for 2.5 h (Fig. 3C), and then infected with HIV-1 for 2 h in the continuous presence of the drugs. Following infection, cell-free virus and the drugs were washed away. Cells were resuspended in fresh medium and cultured in the absence of drugs for 2 days, at which point the GFP-positive cells were measured by flow cytometry. As shown in Fig. 3B, treatment of Rev-CEM cells with TX resulted in an almost 2-fold (from 4.6% to 8.8%) enhancement of HIV infection compared to the results with untreated, HIV-

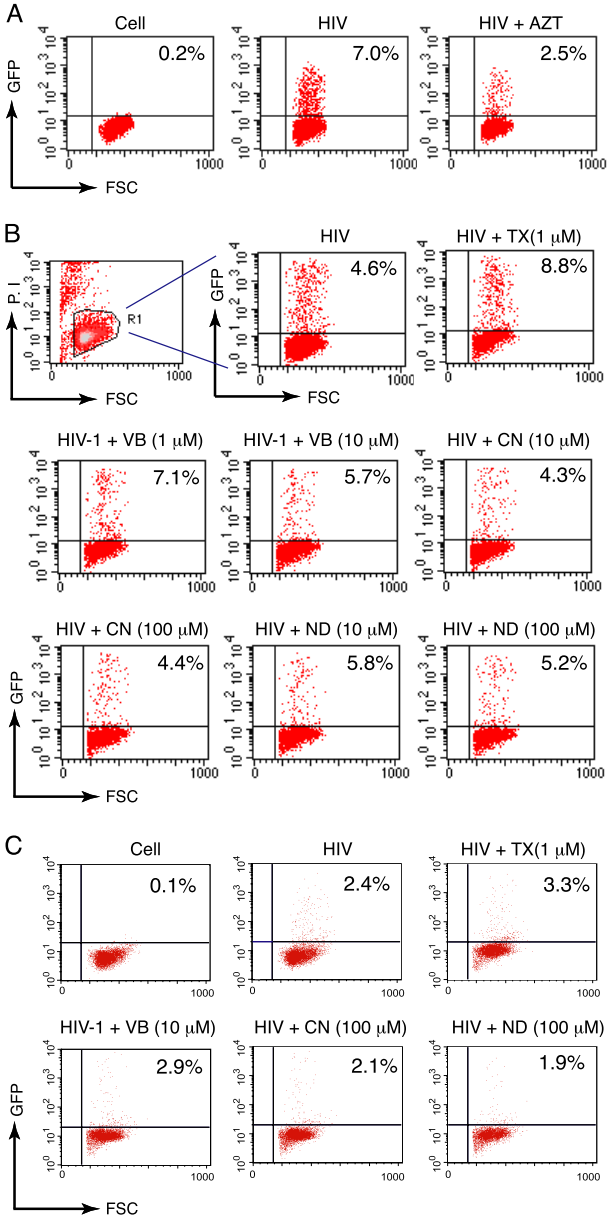

FIG. 3. Effects of microtubule modulators on HIV-1 infection of Rev-CEM cells. (A) Measurement of zidovudine (AZT) inhibition of HIV-1 replication in Rev-CEM cells. For controls, cells were pretreated with 50 μM AZT or medium and subsequently infected with a single-cycle virus, HIV-1(Env) (40) (546 ng p24 per 0.2 million cells), in the presence of AZT for 2 h. Following infection, cells were washed three times and then cultured in the presence of AZT. Viral replication was monitored by flow cytometry analysis of HIV-dependent GFP expression at 48 h (20,000 cells analyzed per sample). Propidum iodide (PI) was added to the cell suspension prior to flow cytometry. Viable cells were gated (R1) based on low PI staining and cell size (FSC). GFP expression within the viable cell population (R1) was measured. The GFP percentages are shown. (B) An experiment similar to that described for panel A but using cells pretreated with TX, VB, CN, or ND at the indicated dosages for 30 min, followed by infection with a single-cycle HIV-1(Env) (228 ng p24 per 0.2 million cells) for 2 h in the continuous presence of these drugs. Following infection, cells were washed three times with medium and then cultured in the absence of drugs. Viral replication was monitored by flow cytometry analysis of HIV-dependent GFP expression within the viable cell population (R1) at 48 h. (C) An experiment similar to that described for panel B but with a new batch of the virus and longer drug pretreatment for 2.5 h prior to infection.

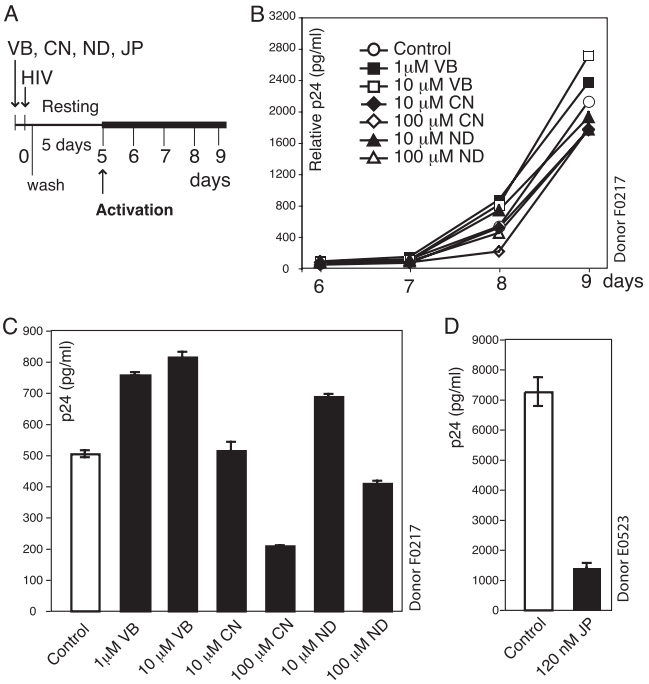

FIG. 4. Effects of microtubule modulators on HIV-1 infection of resting CD4 T cells. (A) Resting CD4 T cells were treated with microtubule modulator VB, CN, or ND for 30 min and then infected with HIV-1 NL4-3 (600 ng p24 per 10^6 cells) for 2 h in the continuous presence of the drugs. Following infection, cells were washed twice with medium and then cultured in the absence of the drugs for 5 days. Cells were activated at day 5 with anti-CD3/CD28 magnetic beads (4 beads per cell), and viral replication was measured by p24 release. (B) Extracellular p24 released from day 6 to 9 postinfection. (C) Average p24 level at day 8 postinfection measured in triplicate. (D) For comparison, resting T cells were also similarly pretreated with 120 nM jasplakinolide (JP) and then infected with HIV-1 (225 ng p24 per 10^6 cells). Shown are the extracellular p24 levels at day 9 postinfection.

1-infected cells. Surprisingly, treatment of Rev-CEM cells with VB also slightly enhanced HIV-1 infection, despite considerable collapse of the microtubule network and high drug cytotoxicity. CN treatment did not significantly inhibit or enhance HIV-1 infection, whereas ND treatment slightly enhanced HIV-1 infection. A similar lack of inhibition was also seen when cells were pretreated for longer time (2.5 h) (Fig. 3C). Based on these data, it appears that during the first 2 h of HIV-1 infection, intact microtubules may not be required for productive HIV-1 infection of transformed T cells.

We further investigated whether these microtubule modulators would affect HIV-1 latent infection of resting CD4 T cells in blood. Cells were similarly pretreated with the drugs and then infected with HIV-1 for 2 h in the presence of the drugs (Fig. 4A). Cell-free virus and the drugs were washed away following infection. Infected cells were resuspended in fresh medium and cultured for 5 days in the absence of the drugs and stimulation. During this period, no viral replication occurs. However, HIV replication remains inducible upon T cell activation. Cells were subsequently activated at day 5 with CD3/CD28 bead stimulation (4 beads per cell), and HIV replication was monitored by extracellular p24 release. As shown in Fig. 4B and C, none of the microtubule modulators except 100 μM CN inhibited HIV-1 latent infection of resting CD4 T cells. The fact that 100 μM CN inhibited HIV-1 infection probably resulted from its general cytotoxicity at such a high dosage rather than from specific microtubule disruption, since 10 μM CN had no effect on HIV-1 latent infection of resting T cells despite its ability to disrupt microtubules. Treatment with both dosages of VB and with 10 μM ND actually slightly enhanced HIV-1 infection, although the reason is unknown. Similar enhancements of HIV-1 replication by TX, VB, and ND were also observed in the infection of Rev-CEM cells (Fig. 3B). Perhaps microtubule modulation promotes intracellular survival of HIV-1 that enters via endocytosis. HIV-1 virions which enter cells through the endocytic pathway are usually destroyed in late endosomes. Microtubule disruption or stabilization could block the transport of endosome carrier vesicles to late endosomes (3). It is known that treatment of cells with pharmacological agents that block viral degradation in late endosomes greatly enhances HIV infection (15).

The lack of inhibition of HIV infection by microtubule drugs could result from possible reassembly of the microtubule network following virus and drug removal. However, it has been shown that early signals mediated from the viral envelope protein play a critical role in viral nuclear migration (48). A significant fraction of viral DNA migrated into the nucleus of resting T cells during the first 2 h, concurrent with the gp120-mediated signaling events (48). When input viruses were removed following infection, viral nuclear migration was decreased in the absence of gp120 signaling, leading to the gradual decay of residual viral DNA (48, 49). Thus, even with possible reassembly of the microtubule network at a later time, viral nuclear migration may not occur in the absence of gp120 signaling.

For a comparison, we also identically treated resting CD4 T cells with an actin inhibitor, jasplakinolide (JP) (8). In contrast to the general lack of inhibition of viral infection by microtubule inhibitors, JP effectively inhibited viral latent infection of resting CD4 T cells at 120 nM (Fig. 4D) (48). These data suggest that intact microtubules may not be required, at least during the first 2 h of HIV-1 infection of resting CD4 T cells, whereas early actin dynamics are critical (9, 48). Actin-dependent intracellular migration has been demonstrated recently in certain viruses such as the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV). AcMNPV utilizes exclusively actin filaments, not microtubules, for nuclear migration (31). In HIV-1 infection, multiple viral proteins in the preintegration complex (PIC) are known to directly interact with actin (13, 16, 22, 30, 35, 40, 43), suggesting possible anchorage of PIC onto the cortical actin for reverse transcription and intracellular migration (9, 23, 47, 48).

Although our study of the microtubule modulators does not appear to support an essential role of microtubules in the early phases of HIV infection of T cells, we do not exclude the importance of the microtubule network for late infection events, such as viral assembly, budding, and spread (20). In addition, our data also do not exclude the involvement of microtubules in the infection of adherent cells such as macrophages, which have more extensive microtubule networks than CD4 T cells.

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